The *Saccharomyces cerevisiae* flavodoxin-like proteins Ycp4 and Rfs1 play a role in stress response and in the regulation of genes related to metabolism.

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ABSTRACT

SPI1 is a gene whose expression responds to many environmental stimuli, including entry into stationary phase. We have performed a screening to identify genes that activate *SPI1* promoter when over expressed. The phosphatidylinositol-4-phosphate 5-kinase gene *MSS4* was identified as a positive activator of *SPI1*. Another *SPI1* transcriptional regulator isolated was the flavodoxin-like gene *YCP4*. *YCP4* and its homolog *RFS1* regulate the expression of many genes during the late stages of growth. The double deletion mutant in *YCP4* and its homolog *RFS1* has an impact on gene expression related to metabolism by increasing the expression of genes involved in hexose transport and glycolysis, and decreasing expression of genes of amino acid metabolism pathways. Genes related to mating and response to pheromone show a decreased expression in the double mutant, while transcription of genes involved in translational elongation is increased. Deletion of these genes, together with the third member of the family, *PST2*, has a complex effect on the stress response. For instance double mutant *ycp4*Δ*rfs1*Δ has an increased response to oxidative stress, but a decreased tolerance to cell-damaging agent SDS. Additionally, this mutation affects chronological aging and slightly increases fermentative capacity.

Key words: *S. cerevisiae*, stress, flavodoxin-like, *YCP4, RFS1*

INTRODUCTION

In media with glucose as the sole carbon source and an excess of nutrients, biomass grows exponentially, metabolism is fermentative, and respiratory metabolism genes are repressed. When levels of the fermentable carbon source decrease, the cell enters into a transition phase between fermentative and respiratory metabolism, called the diauxic shift. This is followed by the post-diauxic growth phase, characterized by respiratory metabolism, transcription of the genes repressed by glucose, and adaptation to the use of ethanol, acetate and other fermentation products as a source of energy (Herman, 2002). Some authors make a distinction between diauxic change and entry into stationary phase (Reinders *et al*., 1998), based mainly on the type of medium in which cells are growing. Yeast growing in rich medium, such as YPD, can survive for several weeks and have a prolonged period of diauxic change. In contrast, yeasts growing in complete synthetic medium (SC) enter into a high metabolism post-diauxic phase that leads to shorter lifespan. This medium is therefore preferentially used to monitor chronological lifespan in studies focused on cell aging (Fabrizio and Longo, 2003).

SPI1 encodes a serine/threonine-rich protein anchored to the cell wall by glycosylphosphatidylinositol (GPI) (Kapteyn *et al*., 1999). It has been shown that is important in cellular resistance to herbicides, wall lytic enzymes, food preservatives and weak lipophilic acids (Simoes *et al*., 2006). We have shown that *SPI1* deletion causes a decrease in cell viability along the growth curve, and increased sensitivity to heat shock, high ethanol concentration and extreme pH values (Cardona *et al*., 2009). *SPI1* is induced in several adverse conditions, particularly under oxidative, heat, ethanol, acetaldehyde and hyperosmotic stresses, lack of nitrogen and amino acids, and acidic or basic pH, and its expression is particularly high during the diauxic change, stationary phase and nutrient starvation in laboratory and wine-making conditions (Gasch *et al*., 2000; Puig and Perez-Ortin, 2000). According to this, its promoter has been used successfully for the overexpression of stress response genes in the later stages of winemaking (Cardona *et al*., 2007). Some facts about the transcriptional regulation of this gene are known. Its induction by glucose starvation is partly dependent on Msn2/4 (Puig and Pérez-Ortín, 2000), its transcription is repressed by Srb10 in aerobiosis (Nuñez *et al*., 2007), and expression in post-diauxic phase is regulated by the ubiquitin ligase Rsp5 (Cardona *et al*., 2009).

Flavodoxins are proteins involved with electron transfer in several processes in photosynthetic and non photosynthetic bacteria and algae, playing an adaptive role allowing survival and reproduction under adverse conditions (Sancho, 2006). Their redox activity occurs via binding to flavin mononucleotide (FMN). Flavodoxins are not present in higher or non photosynthetic eukaryotes, but their relationship with NAD(P)H:quinone-oxidoreductases in these organisms is clear (Carey *et al*., 2007). There is a growing family of flavodoxin like proteins that share a typical α/β twisted open-sheet fold, with an additional α/β unit (Grandory and Carey, 1994). The prototypic protein of this family is the *Escherichia coli* Trp-repressor binding protein WrbA. This protein binds FMN, and is able to form tetramers but has no effect on the DNA binding properties of its partner, the Trp repressor (Grandori *et al*.,

1998). Additionally, it plays a role in heat shock and oxidative stress protection (Daher *et al*., 2005). The *Schizosaccharomyces pombe* ortholog, Uhp1, binds to histones, is associated with silent chromatin and regulates mating-type loci gene expression (Naresh *et al*., 2003).

In the *S. cerevisiae* genome, three WrbA orthologs have been found: *PST2 YCP4* and *RFS1* (Grandori and Carey, 1994). *PST2* is a mating-type regulated gene whose deletion suppresses the DNA repair defect of a *RAD55* mutant, a phenotype that is shared with *RFS1* but not with *YCP4* mutant (Valencia-Burton *et al*., 2006). Pst2 also associates with chromatin in a non-random fashion. *S. cerevisae* gene *PST2* is induced by oxidative stress in a Skn7 and Yap1 dependent manner (Lee *et al*., 1999). Pst2 is secreted in cell wall regenerating protoplasts (Pardo *et al*, 2000) and is important for the exit from stationary phase (Martínez *et al*., 2004). *RFS1* transcription is activated in galactose based medium in an Msn2/4 dependent manner (Lai *et al*., 2005). Ycp4 is palmitoylated, a post-translational modification typical of membrane-binding proteins involved in signal transduction and vesicular sorting (Roth *et al*., 2006). Global analyses of protein-protein interactions in yeast have shown a complex network of interactions (Uetz *et al*., 2000; Collins *et al*., 2007). Pst2 binds to itself and the other two members of the family, and Ycp4 and Rfs1 are able to interact with each other.

There is controversy surrounding the cellular localization of these proteins. Ycp4 was detected in highly purified mitochondria in high-throughput studies (Sickmann *et al*., 2003), it was also detected in the plasma membrane proteome (Delom *et al*., 2006), and the GFP fusion is a cytosolic protein that gives a punctuate pattern of distribution (Huh *et al*., 2003). This punctuate pattern has been related to the formation of certain membrane microdomains (Grossman *et al*., 2008) to which the three flavodoxin-like proteins are attached from the cytosolic side. Rfs1 has a very similar distribution, however it has not been found in the mitochondria.

In this work we carried out a genetic screening to identify novel transcriptional regulation during the late stages of growth, using the *SPI1* gene as a reporter. We have found several genes that affect positively *SPI1* expression, including genes involved with tryptophan metabolism, the phosphatidylinositol-4-phosphate (PPI4P) 5-kinase *MSS4* (which participates in actin cytoskeleton organization and cell morphogenesis; Desrivieres, 1998), the glycerol kinase gene *HOR2* and the flavodoxin-like gene *YCP4*. We have shown that *YCP4* and its homolog *RFS1* are genes relevant to stress tolerance and that they regulate *SPI1* expression at the entry into stationary phase. Furthermore both genes play a common role in the mRNA expression of a variety of metabolic processes, repressing genes involved in carbon metabolism and activating genes related to nitrogen metabolism. The repression of a large number of genes involved in mating and response to pheromones is seen in the double *ycp4*Δ*rfs1*Δ mutant.

MATERIALS AND METHODS

Yeast strains and growth conditions

The yeast strains, plasmids and oligonucleotides used in this work are listed in Supplementary Tables 1A, 1B and 1C respectively.

For yeast growth, YPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose) and SC (0.17% (w/v) yeast nitrogen base, 0.5% (w/v) ammonium sulphate, 2% (w/v) glucose, 0,2% drop-out mix of all supplements, unless indicated) were used (Adams *et al*., 1997). X-gal plates were prepared by mixing 800 mL of a heat sterilized solution composed of: yeast nitrogen base without amino acids 0.8375% (p/v), glucose 2%, drop out mix without uracil 0.25% and agar 2%; 200 mL of a heat sterilized phosphate buffer solution (KH_2PO_4 1M,

$(NH_4)_2SO_4$ 0.15M, KOH 0.75 M, pH 7) and 1 mL of a filter-sterilized X-gal (5-bromo-4-cloro-3-indolil-β-D-galactopiranoside) solution (40 mg/mL). Cultures were incubated at 30º C with shaking.

To analyze stress resistance, cells from exponential phase cultures in YPD medium were treated by the following conditions: a shift to YPD containing 1 M KCl (osmotic stress) or SDS 0.05% (cell wall damage stress) and incubation for 30 minutes at 42ºC. In all cases, cell viability was determined by counting the number of colonies growing on YPD plates following appropriate dilutions of cultures. To analyze resistance to oxidative stress, the diameter of the growth inhibition region produced by a paper disk with 10 µL of 33% H_2O_2 on stationary phase cultures on YPD plates was measured (Carrasco *et al.*, 2001).

All the experiments were carried out at least in triplicate and the mean, standard deviation and statistical significance (student's t-test, unequal variance, p-value for one tail $\leq$ 0.05) were calculated using Microsoft Office Excel.

Construction of plasmids and yeast strains

Ycp4 and Rfs1 proteins were C-terminally tagged with HA by cloning into plasmid pHAC33 (a gift from M. N. Hall) adding *Xba*I and *Sal*I restriction sites to the oligonucleotides used (see Table 1C of Supplementary Material), and transformed into strain YPH499.

RFS1 deletion was performed with a *LEU2* cassette following the PCR-based gene modification method described by Longtine *et al.* (1998) using the appropriate oligonucleotides (See Table 1C of the Supplementary Material). Yeast transformations were carried out by the lithium acetate method (Gietz and Woods, 2002)

Genetic screening

To detect intense or early blue colonies, plates containing SC-URA-LEU + Xgal were used. *W303-1a* strain was co-transformed with the *SPI1-lacZ* (Cardona *et al*., 2009) fusion and with a library constructed in the episomal plasmid Yep13 (a gift from J.C. Igual). The plasmids of the library were sequenced from both sides of the insert.

Expression of the *SPI1-lacZ* fusion expression was quantified by measuring β-galactosidase activity in liquid medium using the method of permeabilized cells with ONPG as a substrate as described by Adams *et al.* (1997).

RNA analysis

RNA isolation and quantification were carried out as previously described (Carrasco *et al.*, 2001). Analysis of the expression of the *SPI1* gene by northern blotting was carried out as previously described (Cardona *et al*., 2009).

For microarray experiments, cDNA preparation, labelling, hybridization and data analysis were carried out as described in Jiménez-Martí *et al*. (2008) The combinations for hybridization Cy3-Cy5 were: WTa-*ycp4*Δ*rfs1*Δa, *ycp4*Δ*rfs1*Δb-WTb, where the a and b samples were obtained from two independent cultures of each strain. The intensity obtained in each channel for each pair of microarrays was normalized by Lowess (Yang *et al.*, 2002). The over-representation of categories containing functionally related genes in each strain was statistically analyzed using the "FuncAssociate 2.0" tool (http://llama.mshri.on.ca/funcassociate/).

Catalase activity, fermentative capacity and chronological ageing methods.

The catalase activity was determined in whole cell extracts by measuring the hydrogen peroxide consumption according to Jakubowsky *et al*. (2000).

The fermentative capacity was measured on SC medium. 250 mL bottles were filled with 100 mL of medium, and 10^7 cells mL^{-1} were inoculated from a preculture grown overnight in SC. Cells were counted with a hemocytometer chamber. Cultures were shaken at 65 rpm in a water bath at 30ºC. CO_2 production was measured every 20 minutes with a Fermograph II (Atto Co. Ltd., Tokyo, Japan). The fermentative capacity was expressed as mL of CO_2 produced per number of cells over time.

Chronological ageing was measured according to Fabrizio and Longo (2003). Cells grown overnight in YPD were diluted in SC to an initial density of 10^6 cells/ml. After two days of growth (when cells do not divide any more, taken as the initial point of aging) cells samples were harvested, diluted and plated, and c.f.u. were counted. The ratio of cells able to form a colony compared to day 0 is measured with respect to time.

Microscopy experiments

Indirect inmunofluorescence microscopy studies were carried out as described in Queralt and Igual (2005) using strains transformed with HA fusion of Ycp4 and Rfs1, and growth in SC-URA in exponential phase. The primary antibody used was anti-HA (3F10, Roche Ref 1867423) and the secondary Alexa 546-labeled anti-rat (Molecular Probes).

RESULTS

Screening for multicopy inducers of SPI1 expression

In order to detect regulators of *SPI1* expression, we previously developed a plasmid containing a *SPI1* promoter fusion to *E. coli lacZ* (*SPI1-lacZ*; Cardona *et al*., 2009). In this study we transformed the wild type *W303-1a* strain containing the reporter plasmid with a genomic library on a multicopy plasmid and selected the transformants that lead to colonies developing an early blue colour when replica plated on selective SC-Xgal plates. This method allows identification of positive activators of the *SPI1* promoter. Selected clones were checked by measuring the β-galactosidase activity of liquid cultures from reporter strains retransformed with the plasmid isolated from the screening experiment. The eight positive plasmids with higher β-galactosidase activity are shown in Table 1.

In order to assess the effect of the over expression of selected genes on the *SPI1* mRNA levels and taking into account potential effects on translation or stability of the *SPI1-lacZ* fusion, northern blot analyses were carried out on cells from post-diauxic phase and glucose deprivation conditions (Fig. 1A and B, respectively).

Plasmids 1 and 54 contain an overlapping DNA region that includes the *SOK1* and *TRP1* genes. We have previously shown that tryptophan metabolism affects *SPI1* expression and indeed a multicopy or centromeric plasmid containing the *TRP1* gene (involved in the biosynthesis of tryptophan) is able to activate the *SPI1* gene in a *trp1-* background (Cardona *et al*., 2009). As seen in Fig. 1A, the addition on multicopy of *TRP1* gene or the high affinity tryptophan permease *TAT2* cause an increase on *SPI1* expression on postdiauxic phase.

Plasmid 13 contains a single gene, *HOR2* (*GPP2)*, that codes for a glycerol-3-phosphatase. Its over expression increases *SPI1* levels in postdiauxic phase, however this effect is less pronounced under glucose starvation conditions (Fig. 1). The knock-out *hor2* mutant has no effect on *SPI1* expression (Supplementary Fig. 1). This may be due to the fact that the functionally redundant *GPP1* gene may complement this mutation. We do not currently

understand the molecular link between glycerol synthesis and *SPI1* expression although it may be related to the fact that *SPI1* being an osmotic stress induced gene (Gasch *et al*., 2000), it could react to the hyperosmotic stress produced by the rise of the glycerol concentration.

Plasmid 228 contained *MSS4,* that encodes an interesting regulatory protein, a PPI4 5-kinase involved in actin cytoskeleton organization and cell morphogenesis (Desrivieres, 1998). A plasmid containing *MSS4* (228) is able to activate *SPI1* gene expression in cells from both diauxic phase and glucose deprivation conditions (Fig. 1). *MSS4* is an essential gene; therefore we used a conditional mutant to characterise its effect on *SPI1* mRNA levels by northern blot analysis (Fig. 1C). *SPI1* transcripts decrease after 6 hours at the non-permissive temperature in the mutant strain, indicating a role of Mss4 in the regulation of this cell wall gene.

We did not further investigate the genes contained in plasmids 87 and 533 although they induce an activation of *SPI1* under postdiauxic phase conditions (see Fig. 1). Finally, we found two plasmids, 116 and 548, overlapping in a DNA region including the mitochondrial ribosomal protein gene *MRPL32* and the flavodoxin-like protein gene *YCP4*. We subcloned the *YCP4* gene in a multicopy plasmid and found that this one is the gene that was causing the *SPI1* induction when over-expressed in cells from the postdiauxic phase of growth and glucose starvation (Figs. 1A and 1B). The remainder of this study will focus on the role of Ycp4 and its homologues in gene expression.

Expression of SPI1 is affected by YCP4 and RFS1

We used the *SPI1-lacZ* fusion to investigate the effect of deleting the *YCP4* gene, throughout the growth curve (Fig. 2A). The *SPI1* promoter expression is not affected in the initial stages of growth but there is a clear reduction in the *ycp4* deletion mutant in the latter stages. This result is confirmed by northern blot (Fig. 2B). Therefore Ycp4 regulates *SPI1* mRNA

levels at the advanced stages of growth. There are three flavodoxin-like genes in the *S. cerevisiae* genome: *YCP4, PST2* and *RFS1* (see Supplementary Fig. 2). The deletion of *RFS1*, but not *PST2*, results in a similar decrease of *SPI1* mRNA levels as tested by northern blot (Fig. 2B). We constructed the double *ycp4*Δ*rfs1*Δ mutant that showed a loss of *SPI1* induction at advanced phases of growth (see also Fig. 2B). Interestingly, testing *SPI1* expression using the *SPI1-lacZ* fusion was only possible using the *ycp4*Δ strain due to the poor growth of the mutants *pst2*Δ and *rfs1*Δ transformed with *SPI1-lacZ* fusion plasmid (data not shown). This is caused by the loss in those mutants of the reporter plasmid in a specific manner relatively to the empty plasmid (Supplementary Fig. 3). That may indicate a direct effect of both proteins at the DNA level in a *SPI1* promoter specific fashion.

*Global study of gene expression in ycp4*Δ*rfs1*Δ *at the diauxic shift*

In order to gain a wider view on the effect of the flavodoxin-like proteins on gene expression we performed a global analysis on *ycp4*Δ and *ycp4*Δ*rfs1*Δ mutant strains compared to the wild type strain in minimal complete SC medium (to avoid growth differences due to the *LEU2* gene present in the *ycp4*Δ*rfs1*Δ deletion strain) at 8 hours following the inoculation at 0.3 OD_{600} units, a point during growth corresponding to the greatest difference in *SPI1* expression between wild type and *ycp4*Δ strains (see Fig. 2A). *YCP4* deletion results in the change of expression of only a few genes (data not shown), suggesting that its function may be replaced by the other flavodoxin-like proteins. In the case of the double mutant, more changes were observed (Supplementary Table 2). Using a cut off value of a 2-fold change in gene expression, 199 genes were up-regulated and 268 down-regulated than in the wild type strain, and gene ontology (GO) categories were found in both cases (Table 2 and Supplementary Tables 3 and 4).

Of the up-regulated genes in the mutant, most GO categories are related to carbohydrate metabolic processes such hexose transport, glycolysis and alcohol metabolism. Strikingly, among the genes with a higher expression most of the high-affinity glucose transporters are present, for instance *HXT2*, *6* and *7* (Ozcan and Johnston, 1995). Moreover, the main glucose phosphorylating enzymes genes show increased mRNA levels, as is the case of *HXK1* (5.85 times), *HXK2* (5.06 times), *EMI2* (6.22 times) and, to a lesser extent, *GLK1* (1.74 times). Some genes along the glycolytic pathway such *FBA1*, *TDH2*, *PGK1*, *GMP1* and *GMP2* are also targeted. These results indicate that the flavodoxin-like proteins contribute somehow to the control of the repression of the genes involved in the hexose metabolism, particularly in the first steps of glucose catabolism (See Supplementary Fig. 4). It is intriguing that many translation elongation factors, both cytosolic and mitochondrial, are slightly up-regulated in the mutant indicating a potential role of these proteins in gene expression at the post-transcriptional level.

The down-regulated genes show enrichment in GO categories related to mating and response to pheromones, and in many categories of amino acid metabolic processes (Table 2 and Supplementary Table 3). Of the genes within the pheromone signalling categories it is worth mentioning the down-regulation of mating factors, both α (*MFα2* 5.69 fold and *MFα1* 2.89 fold) and a (*MFA1* 2.34 fold and *MFA2* 2.70 fold), both pheromone receptors (*STE2* 7.86 fold and *STE3* 3.45 fold), the last MAP kinase of the cascade, *FUS3* (4.06 fold) and the main transcription factor of the pathway, *STE12* (3.75 fold). The enrichment found in our microarray data in the functional categories of amino acid biosynthesis, particularly of the arginine, sulfur amino acids (including the sulphate assimilation pathway), aspartate, glutamate and serine, suggest that the flavodoxin-like proteins Ycp4 and Rfs1 could have a positive effect on nitrogen metabolism and, according to the data described above, a negative effect on carbon metabolism (see Supplementary Fig. 4).

The results obtained with arrays technology have been confirmed by means of a semiquantitative RT-PCR analysis with a selection of the genes whose transcription is affected in the double mutant arrays, using the *ACT1* gene as a housekeeping control. The results are shown in Supplementary Fig. 5. Genes involved in the carbohydrate metabolism (such as *HXT6/7*, *HXK2* and *GAC1*) and the amino acid metabolism (*MET10*, *STR3* and *ARG4*) among others were selected for analysis.

Flavodoxin-like proteins are involved in stress tolerance, aging and fermentative capacity in S. cerevisiae

Due to the involvement of flavodoxin-like proteins in stress resistance in *E. coli*, we tested the effect of deleting each one of the yeast's three flavodoxin-like genes (and also of the double deletion *ycp4*Δ*rfs1*Δ) on viability following exposure to several adverse conditions. Fig. 3 shows the viability of the wild type and mutant strains after oxidative and osmotic stresses, as well as cell wall damage. There was no significant difference in heat shock stress in the tested conditions (data not shown). The mutant *rfs1*Δ strain is the only one that is significantly sensitive to H_2O_2 stress (Fig. 3A). That suggests that even though the flavodoxin fold may have a redox role, this may not be relevant in all tested proteins. Surprisingly double mutant *ycp4*Δ*rfs1*Δ is more tolerant to oxidative insult. Osmotic stress caused by KCl reduced viability of mutants *pst2*Δ and *rfs1*Δ, but not of *ycp4*Δ (Fig. 3B). The double mutant restores the sensitivity of the *rfs1*Δ to wild-type tolerance. Finally the effect of cell wall damaging agent SDS (Fig. 3C) shows that the cell wall is more damaged in *pst2*Δ and *rfs1*Δ mutants, but in this case the double mutant *ycp4*Δ*rfs1*Δ is similarly sensitive to the single *rfs1*Δ mutant. Therefore Rfs1 seems to play a clear role in stress tolerance via a complex network of interactions with its homologue Ycp4.

As seen in Fig. 3A, the double mutant *ycp4*Δ*rfs1*Δ has increased tolerance to hydrogen peroxide. The microarray experiments show an increase of 2.52 fold in the cytosolic catalase *CTT1* expression (see Supplementary table 2). We tested the catalase activity of this mutant in stationary phase in the presence or absence con H_2O_2 (see Fig. 4A). In these conditions, the oxidative insult cause a significant increase of catalase activity in the double mutant, but not in the wild type strain, suggesting a role of catalase in providing a better tolerance to oxidative stress.

We have identified a clear role of the Ycp4 and Rfs1 proteins in controlling gene expression at late stages of growth. In order to assess their effect on long term survival we tested the chronological lifespan of the double mutant strain, measured as the ability to survive through time in synthetic rich media SC (Fig. 4B) according to the protocol developed by Fabrizio and Longo (2003). The double deletion causes an increase in the short term chronological lifespan, but a decrease in the long term lifespan.

Finally, in order to improve our knowledge of the activity of the double mutant during glucose metabolism we studied the fermentative capacity measured as CO_2 production in SC (Fig. 4C). In accordance to the microarray data, the double mutant is characterised by a slight increase in carbon metabolism, resulting in a faster usage of glucose.

Subcellular localization of flavodoxin-like proteins Ycp4 and Rfs1

As mentioned in the introduction, various studies place the yeast flavodoxin-like proteins in different subcelullar locations. In order to study the localization of Ycp4 and Rfs1 during growth, an immunochemical localization of the HA-tagged proteins was carried out (Fig. 5). During exponential growth, as expected, both proteins show a clear punctuate distribution, with no co-localization with the nuclei (Fig. 5A). During stationary phase the punctuate pattern

remains unchanged for both proteins (Fig. 5B). Therefore the function of Ycp4 and Rfs1 at the latter stages of growth does not depend on a change of sub-cellular localization.

DISCUSSION

We performed a screening to find new positive regulators of *SPI1* transcription. We have identified the *YCP4* gene as a novel regulator of *SPI1* expression. Ycp4, together with its *S. cerevisiae* homologs Pst2 and Rfs1, is a member of a family of proteins that share structural homology to the flavodoxins. Their *E. coli* ortholog, WrbA, is able to bind FMN, has a NAD(P)H-dependent redox activity and reduces quinones, suggesting a role in oxidative stress tolerance (Patridge and Ferry, 2006). There is no evidence of the FMN binding capacity of the *S. cerevisiae* flavodoxin-like proteins. However PbY20, the ortholog of Ycp4 in the fungus *Paracoccidioides brasiliensis,* does indeed bind FMN (Daher *et al*., 2005), suggesting that their role in redox metabolism could be conserved in fungi. Our results show an involvement of these proteins not only in oxidative stress response (see Fig. 3A and 4A) but also in osmotic stress response (see Fig. 3B). The complex pattern of sensitivity to these two kinds of stress conditions depending on the particular single and double mutant considered may be caused by the establishment of different binding combinations between family members. Ycp4 and Rfs1 bind to each other and both to Pst2, that is able to bind to itself. WrbA participates in a dimer-tetramer equilibrium (Grandori *et al*., 1998). Potentially the deletion of each gene may result in replacement by the other members of the family during a given condition, or cause an opposite effect in some cases due to the formation of novel aggregates.

The sensitivity to oxidative stress as well as growth defect at the diauxic shift and stationary phase, could be related with a potential role of these proteins as electron

transporters in maintenance of the redox equilibrium during oxidative stress and at the shift from fermentative to respiratory metabolism. The increased tolerance to oxidative stress of the double mutant *ycp4*Δ*rfs1*Δ (Fig. 3A) could be due to the fact that it posses more catalase activity (Fig. 4A). The sensitivity to other stress conditions, such as SDS or osmotic stress could be due to the altered expression levels of some genes found using microarray analyses (Table 2), at least in the double mutant *ycp4*Δ*rfs1*Δ. Actually, cell wall genes are preferentially less expressed in the double mutant, suggesting that a cell wall defect can lead to impaired viability in cell wall damaging agent as SDS or following a hyperosmotic shock. Another possibility is that the effect on stress response may be post transcriptional and this is supported by the upregulation of genes involved in translation elongation in the double mutant (Table 2), which could influence the cellular response to stimulus. The mechanism of action of these proteins to modulate gene expression remains elusive. As other reports show, we have found that Ycp4 and Rfs1 are located in a punctuate pattern in the cytoplasm (Fig. 5), with no nuclear colocalization. These results suggest that these proteins may act in the early stages of an unknown signal transduction pathway or in a regulatory mechanism at the posttranscriptional level. However Pst2 has been detected bound to chromatin in a non-random fashion (Valencia-Burton *et al*., 2006), indicating that maybe a minor subset of these proteins may be located in the nucleus, modulating gene expression to some extent.

The use of microarrays shows that *YCP4* and *RFS1* have a clear role in the regulation of metabolism at the late stages of growth. The double mutant has increased the carbon metabolism devoted to energy production and storage and decreased the nitrogen metabolism directed to amino acid biosynthesis (Table 2). We found that the mutant strain has a slightly better fermentative capacity (Fig. 4C) that relates to the increase in the expression of genes involved in energy metabolism. The participation of these proteins in some of these processes, for instance in methionine metabolism, is shared with prokaryotic flavodoxins. It is interesting that *E. coli* WrbA was first described as a binding partner of the tryptophan

repressor TrpR (Yang *et al*., 1993). During the stationary phase, cells deficient in the WrbA protein were less capable than wild type to repress the *trp* promoter, suggesting an ancestral role of this family of proteins in amino acid metabolism during late phases of growth. It is worth noting that in our screening of *SPI1* regulators we isolated genes involved in the biosynthesis of tryptophan along with *YCP4*, suggesting a link between protein families.

A significant number of genes related to mating (particularly the silent mating type genes HMLα2, HMLα1 and HMRa2) are repressed in the double mutant. This increase in gene silencing could be the cause of the extended short-term lifespan of the double mutant strain (Fig. 4B), as it is widely accepted that the control of chromatin condensation by proteins like Sir2 is a key mechanism in the prevention of aging (Guarente, 2000). The increased tolerance to oxidative stress (Fig. 3A) may also contribute to extend the mutant lifespan, as both parameters are tightly linked (Fabrizio and Longo, 2003). Besides, alterations in the control of the metabolism like the ones we observe in this double mutant, may also affect cell aging (Jazswinski, 2000), and that could explain the reduction in long-term survival in the double mutant. In relation to the regulation of mating-type genes, we have searched for common transcription factors in the differentially expressed genes from our microarray data using the Yeastract tool (Monteiro *et al*., 2008). We have found that 55,1% of the genes up-regulated with a fold change of at least 2 and 63,5 % of the down-regulated genes have been documented to be regulated directly by Ste12 transcription factor, a well known regulator of genes involved in mating or pseudohyphal/invasive growth pathways (Wong and Dumas, 2010). *STE12* is itself down-regulated in the mutant strain (3.75 times) in our microarray experiment. This transcriptional regulation could be through Pst2, which interacts with Ste50 (Uetz *et al*., 2000), a regulator of MAP kinase Ste11, involved in the signal transduction pathway that activates Ste12 (Kwan *et al*., 2006). Ste12 controls genes related to filamentous growth (Wong and Dumas, 2010) together with the transcriptional factor Tec1 (reduced 4.18 fold in the double mutant). There is no pseudohyphal growth phenotype in our mutant (data

not shown), probably due to the genetic background we are using, but these proteins could be regulators of cell morphology under specific growth conditions.

Chromatin condensation is linked to genomic stability and the mating status of cell can also affect DNA repair (Valencia-Burton *et al*., 2006). *PST2* and *RFS1* deletion suppress the repair defect caused by deletion of *RAD55*, a gene involved in the recombinational repair of double-strand breaks in DNA during vegetative growth and meiosis. The single mutant strains of the flavodoxin-like genes do not lead to DNA repair defects (Valencia-Burton *et al*., 2006). Equally, the double mutant *ycp4*Δ*rfs1*Δ does not show an altered sensitivity to DNA-damaging drugs like hydroxyurea and phleomycin (data not shown). However, when the strains *pst2*Δ and *rfs1*Δ were transformed with the *SPI1-lacZ* plasmid, we observed that the construct (but not the empty plasmid) was lost (See Supplementary Fig. 3), a fact that could indicate a direct relationship with the *SPI1* promoter or the genetic stability of this region of DNA.

Taking in consideration all the results described in this work, it seems that yeast flavodoxin-like proteins are involved in the regulation of gene expression of metabolic pathways and stress response, but probably not as a transcription factors. Further studies would be required for a complete understanding of the role of these proteins in yeast gene expression.

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Table 1. Plasmids isolated by the genetic screening using *SPI1-lacZ* fusion. Genes whose ORF is completely included in the plasmids are listed. Candidate gene: gene that activates *SPI1* when cloned on its own or whose mutation affects *SPI1* expression. nd (not determined)

Plasmid	Gene(s)	Candidate
1	*RED57, SOK1, TRP1*	*TRP1*
13	*HOR2*	*HOR2*
54	*SOK1, TRP1*	*TRP1*
87	*YPR174c, VPS4, BSP1, YPR172w*	nd
116	*MRPL32, YCP4*	*YCP4*
228	*MSS4, YDR210w*	*MSS4*
548	*CDC10*, *MRPL32, YCP4*	*YCP4*
533	*DEG1*, *LOC1*	nd

Table 2. Genes with a differential expression in *ycp4*Δ*rfs1*Δ mutant strain *vs.* the wild-type strain. Selected genes of the key GO categories that are up- or down-regulated in each category are shown, and their induction or repression values are shown in brackets. All genes induced or repressed by a factor of 4, plus selected genes of the significant categories induced or repressed by a factor of 2 are shown.

a) Up-regulated in the mutant

Categories	Genes
Carbohydrate metabolic process	*ADH4 (8.39), GAC1 (7.21), GSY1 (5,90), FBA1 (5,32), PMI40 (4,22), TDH2 (3,55), MDH2 (3,44), GMP1 (3,27), PGK1 (2,84), GIP2 (2,71) GLC3 (2,66), SUC2 (2,49), PGM2 (2,36), PDC5 (2,22) PSA1 (2,16), GND1 (2,07), RPE1 (2,05)*
Hexose transport	*HXT6 (9.06), HXT7 (8.95), HXT4 (7,04), HXT2 (4,04), ITR1 (4,60) MTH1(2.75)*
Hexokinase	*EMI2 (6,22), HXK1 (5,85), HXK2 (5,06), GLK1 (1.76)*
Translation elongation factor activity	*TEF1 (3.24), MEF2 (3.07), EFT1 (2.66), TEF4 (2.47), TEF3 (2.41), MEF1 (2.28)*
Other	*URA1 (7,04), SAH1 (5,87) OLE1 (5,85) CYC7 (5,72) ISF1 (5,50), ADE17 (5,30) HHO1 (5,22) GAS3 (5,20), RNR2 (4,37)*
Unknown	*YNL144c (5,01), YNL195c (4,85), RGI1 (4,64), YNR014w (4,22)*

b) Down-regulated in the mutant

Categories	Genes
Mating and response to pheromone	*AGA1 (29,29), PRM1 (26,95), KAR4 (17,26), FUS1 (9,92), SST2 (9,36), FIG2 (8,66), STE2 (7,86), FUS2 (7,38), AGA2 (7,08), FAR1 (6,73), MFα2(5,67), SAG1 (4,92), HMLα2 (4,51), FUS3 (4,06)*
Amino acid	*CIT2 (12,28) MET17 (9,66), ARG3 (7,70), ARO10 (6,51), MET1 (5,65), STR3*

biosynthetic process	*(5,63), MET10 (5,21), CPA1 (5,20), MET2 (5,10), ECM17 (4,81), ARG5,6 (4,58), BAT2 (4,00)*
Other	*SNZ1 (9,71), PRM5 (6,92), PRM6 (6,76), DLD3 (6,55), YJL213w (5,42), SAG1 (4,92) MCH4 (4,86), BOP3 (4,83), PRM4 (4,76), EEB1 (4,71), YAT12 (4,63), ICY2 (4,58), MUP1 (4,30), SAM3 (4,21),TEC1 (4,18), GIC2 (4,14)*

Fig. 1. Effect of different multicopy plasmids on *SPI1* expression. Plasmids that contain a single gene are named accordingly and plasmids isolated from the screening are named as in Table 1. We analyzed the expression of *SPI1* in minimal medium by northern blot in post-diauxic phase conditions (A; 8h from a DO_{600}=0.3), in conditions of absence of glucose (B; centrifuged a culture of DO_{600}=0.3, resuspended cells in glucose-free SD medium and incubated for 15 min). The data shown correspond to the relative amount of mRNA of *SPI* with

respect to rRNA, in the test strain vs the same strain transformed with the corresponding empty plasmid. C) Quantification of the expression of *SPI1* by northern blot in *mss4* thermosensitive mutant (strain AAY201), after 0, 6 and 9 hours in 37 º C compared to the parental strain AAY201. All experiments were carried out in triplicate and the standard deviation is indicated. Asterisks indicate statistically significant differences (* p-value <0.05, ** p-value <0.005).

Fig. 2. Effect of flavodoxin-like gene deletions on *SPI1* expression. A) *SPI1-lacZ* induction in *ycp4*Δ strain during growth curve in SC medium (initial inocolum at OD_{600}=0.1). B) *SPI1* expression measured by northern blot in control (EXP) and post-diauxic (PD) conditions in single deletion mutants *ycp4*Δ, *pst2*Δ and *rfs1*Δ and *ycp4*Δ*rfs1*Δ double mutant. All experiments were carried out in triplicate and the standard deviation is indicated. Asterisks indicate statistically significant differences (* p-value <0.05, ** p-value <0.005).

Fig. 3. Effect of flavodoxin-like gene deletions on stress resistance. Effect of oxidative stress (A), osmotic stress (B) and SDS resistance (C) in *ycp4*Δ, *pst2*Δ and *rfs1*Δ single deletion mutants and *ycp4*Δ*rfs1*Δ double mutant. Diameter growth inhibition measurement (cm) is shown for oxidative stress, whereas percentage of survival measured by plate-count is shown for osmotic stress and SDS resistance. All experiments were carried out in triplicate and the standard deviation is indicated. Asterisks indicate statistically significant differences (* p-value <0.05, ** p-value <0.005).

Fig. 4. Effect of the *ycp4*Δ*rfs1*Δ mutation on catalase activity, chronological ageing and fermentative capacity. A) Catalase activity of *ycp4*Δ*rfs1*Δ double mutant and its parental

strain. Stationary cultures in YPD were exposed to 0.2 mM of hydrogen peroxide for 1 h and cellular extracts were tested for catalase activity by measuring the consumption of H_2O_2 at A_{240}. B) Chronological aging of *ycp4*Δ*rfs1*Δ double mutant and its parental strain. Viability expressed as percentage ratio of c.f.u. at each point compared with the initial time is shown as a function of time on a logarithmic scale. C) Fermentative capacity was measured as the volume of CO_2 produced per 10^7 cells as a function of time in cultures of the different strains inoculated at an initialOD_{600}=1 in SC medium. All experiments were carried out in triplicate and the standard deviation is indicated. Asterisks indicate statistically significant differences (* p-value <0.05, ** p-value <0.005).

Fig. 5. Subcellular localization of HA-fusions of Ycp4 and Rfs1 by immunocytochemistry. The cellular location of HA-tagged proteins was determined in YPH499 strain transformed with the tagged versions of *YCP4* and *RFS1* on plasmid pHAC33. Cells were collected in exponential (A) and stationary (B) phases of growth on SC-URA and observed with a fluorescence microscopy after immunostaining with a HA-specific antibody (Cy3). Cells were also stained with DAPI to localize nuclei.

Supplementary Fig. 1. Lack of effect of *hor2* deletion on *SPI1* expression. *SPI1* expression tested by northern blot in wild type strain *BY4742* and *hor2*Δ strain under control conditions

(exponential, EXP) and post-diauxic phase (PD) in rich medium. The data shown correspond to the relative amounts of *SPI1* mRNA relative to rRNA, and for the same strain transformed with the corresponding empty plasmid. All experiments were carried out in triplicate and the standard deviation is indicated.

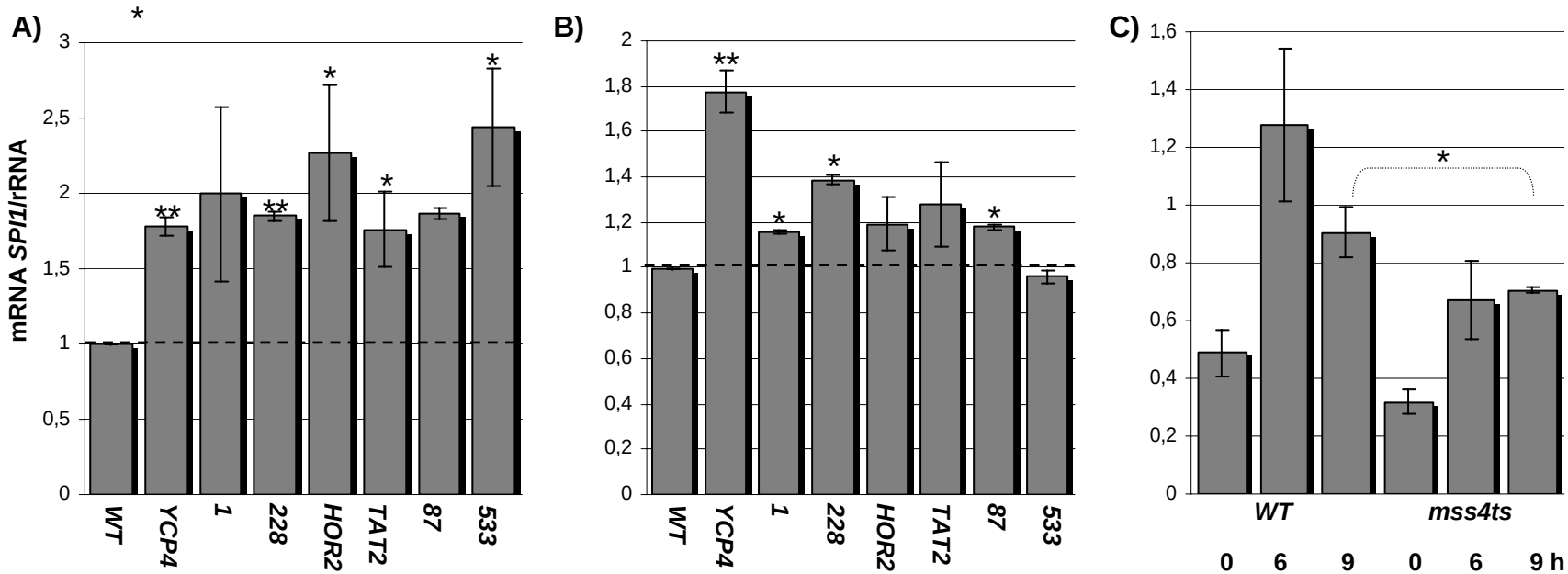
Supplementary Fig. 2. Alignment of flavodoxin-like proteins in *S. cerevesiae* (A) and other organisms (B). The sequences of Ycp4, Pst2 and Rfs1 of *S. cerevisiae* (Sc), PbY20 of *Paracoccidioides brasiliensis* (Pb), Uhp1 of *Schyzosaccharomyces pombe* (Sp) and Wrba of *E. coli* (Ec) were took from NCBI and the program ClustalX 1.83 was used for the alignment. The last 40 amino acids of Ycp4_Sc are omitted as they were not present in other proteins.

Supplementary Fig. 3. Plasmid loss in mutants of flavodoxin-like genes. Yeast strains transformed with YEPlac357 (A) and the *SPI1*-lacZ containing plasmids (B) were grown in nonselective medium (YPD) and dilutions were plated on both selective (SC-URA) and non-selective medium (YPD). The percentage of colonies present on the SC-URA plates compared to the YPD plates is represented. Statistically significant data are indicated by *** (p-value<=0.0005).

Supplementary Fig. 4. Scheme of the results obtained in differential expression between wild type and mutant *ycp4*Δ*rfs1*Δ from microarray experiments. Only the genes related to central cellular metabolism are shown. Green text indicates genes differentially expressed at least two-fold and red text indicates those differentially expressed more than two-fold in the double

mutant compared to wild type. Relationships with the metabolism of amino acids are shown in blue. The red or green boxes highlight the metabolites whose levels are increased or decreased (respectively) in the double null mutant *ycp4*Δ*rfs1*Δ. Purple text show up-regulated genes in the *ycp4*Δ single mutant, whilst red text with an asterisk indicates those overexpressed in both mutants.

Supplementary Fig. 5. Semiquantitative RT-PCR confirmation of selected results obtained by microarray analysis in *ycp4*Δ*rfs1*Δ strain. A representative result of the replicate analysis carried out with RNA from two independent cultures is shown. *ACT1* was used as constitutive gene.


A)
mRNA SPI1/rRNA
3
2,5
2
1,5
1
0,5
0
WT
YCP4
1
228
HOR2
TAT2
87
533
B)
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
WT
YCP4
1
228
HOR2
TAT2
87
533
C)
1,6
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0,4
0,2
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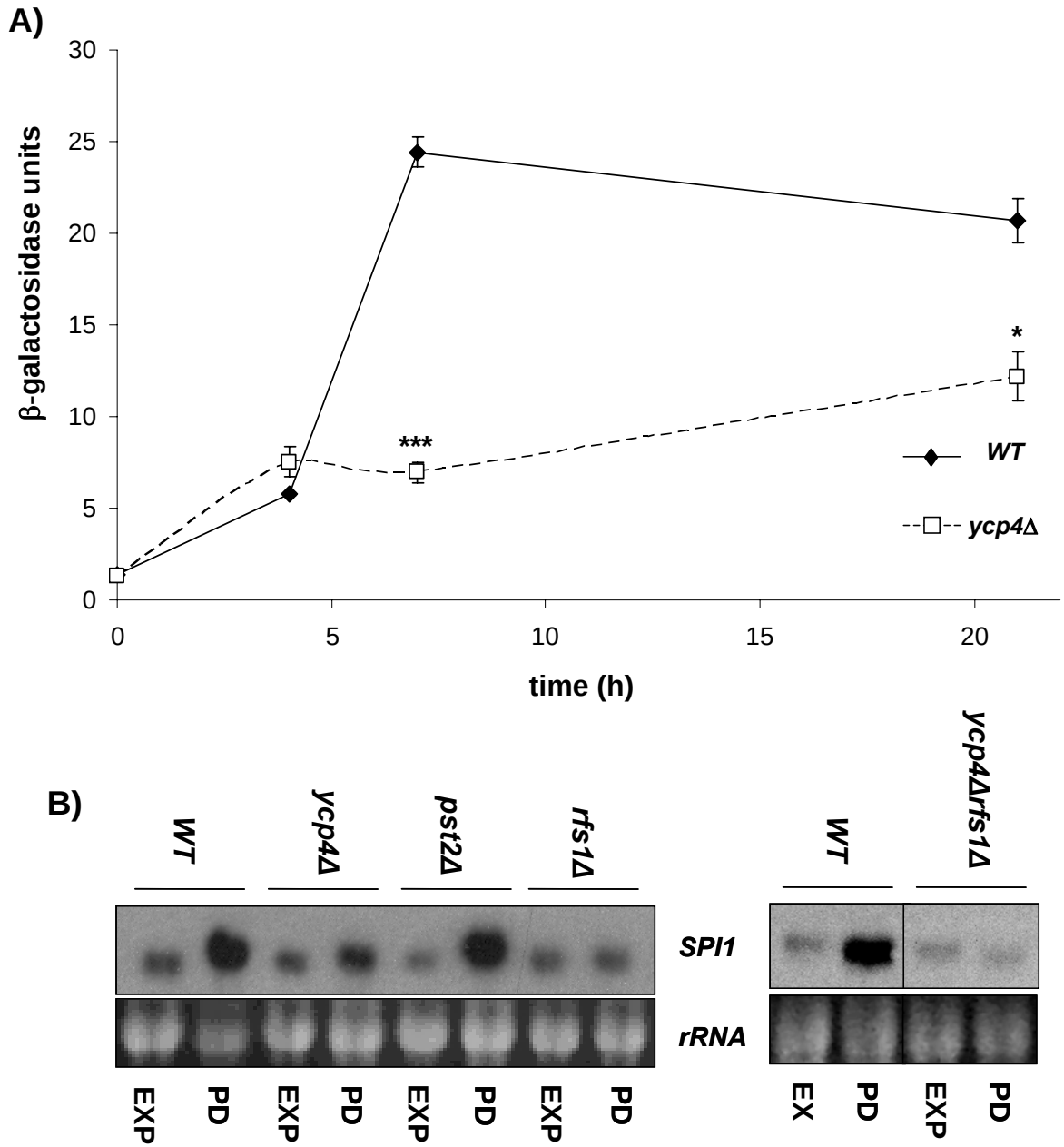
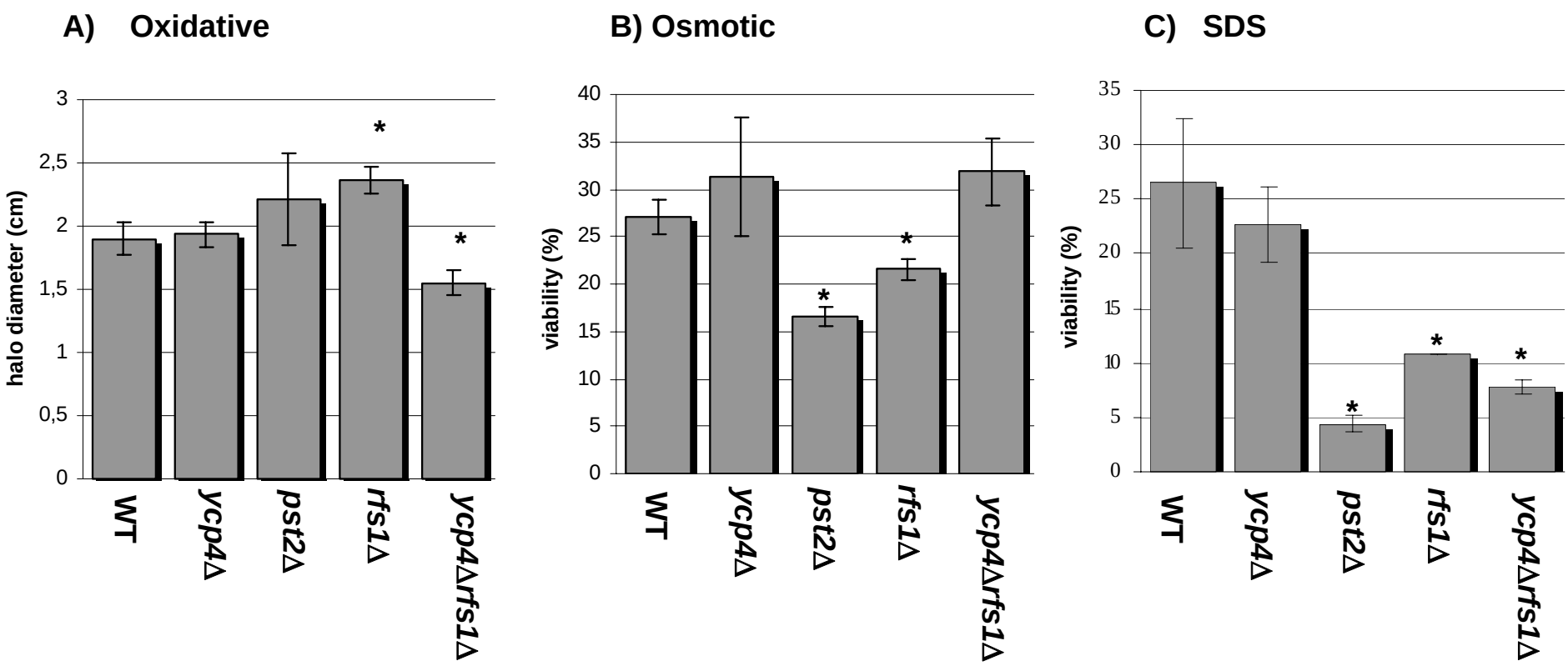
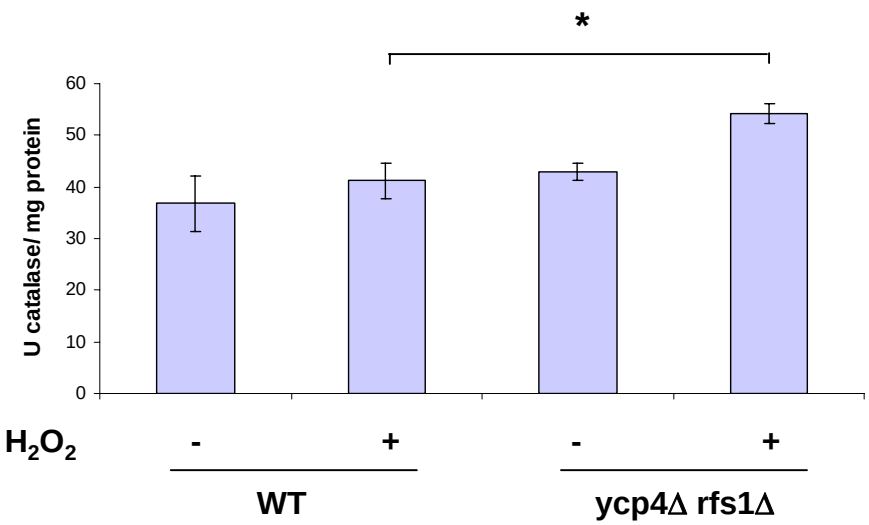
WT
mss4ts
0 6 9 0 6 9 h

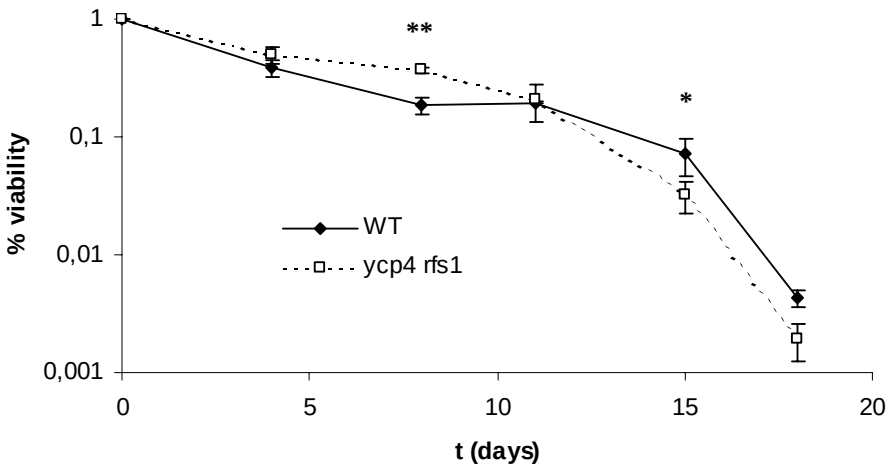

A)
β-galactosidase units
30
25
20
15
10
5
0
0
5
10
15
20
time (h)
***
*
WT
ycp4Δ
B)
WT
ycp4Δ
pst2Δ
rfs1Δ
WT
ycp4Δrfs1Δ
SPI1
rRNA
EXP
PD
EXP
PD
EXP
PD
EXP
PD
EX
PD
EXP
PD


A) Oxidative
halo diameter (cm)
3
2,5
2
1,5
1
0,5
0
*
*
WT
ycp4Δ
pst2Δ
rfs1Δ
ycp4Δrfs1Δ
B) Osmotic
viability (%)
40
35
30
25
20
15
10
5
0
*
*
WT
ycp4Δ
pst2Δ
rfs1Δ
ycp4Δrfs1Δ
C) SDS
viability (%)
35
30
25
20
15
10
5
0
*
*
*
WT
ycp4Δ
pst2Δ
rfs1Δ
ycp4Δrfs1Δ

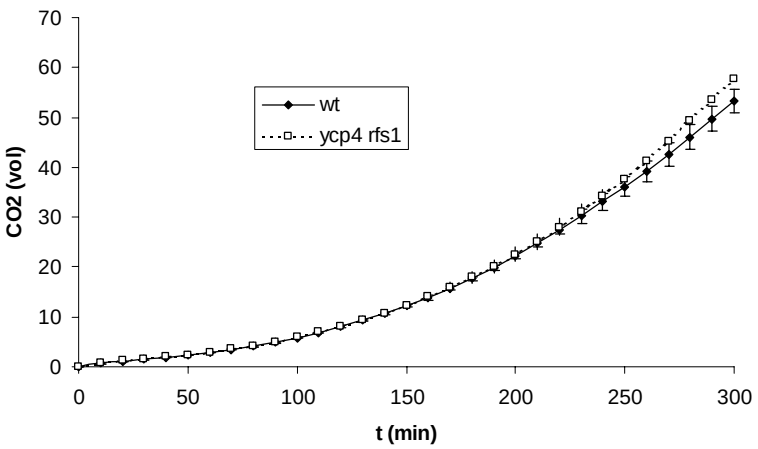
A)


*
U catalase/ mg protein
60
50
40
30
20
10
0
H2O2
-
+
-
+
WT
ycp4Δ rfs1Δ

B)


**
*
% viability
1
0,1
0,01
0,001
WT
ycp4 rfs1
0
5
10
15
20
t (days)

C)


wt
ycp4 rfs1
CO2 (vol)
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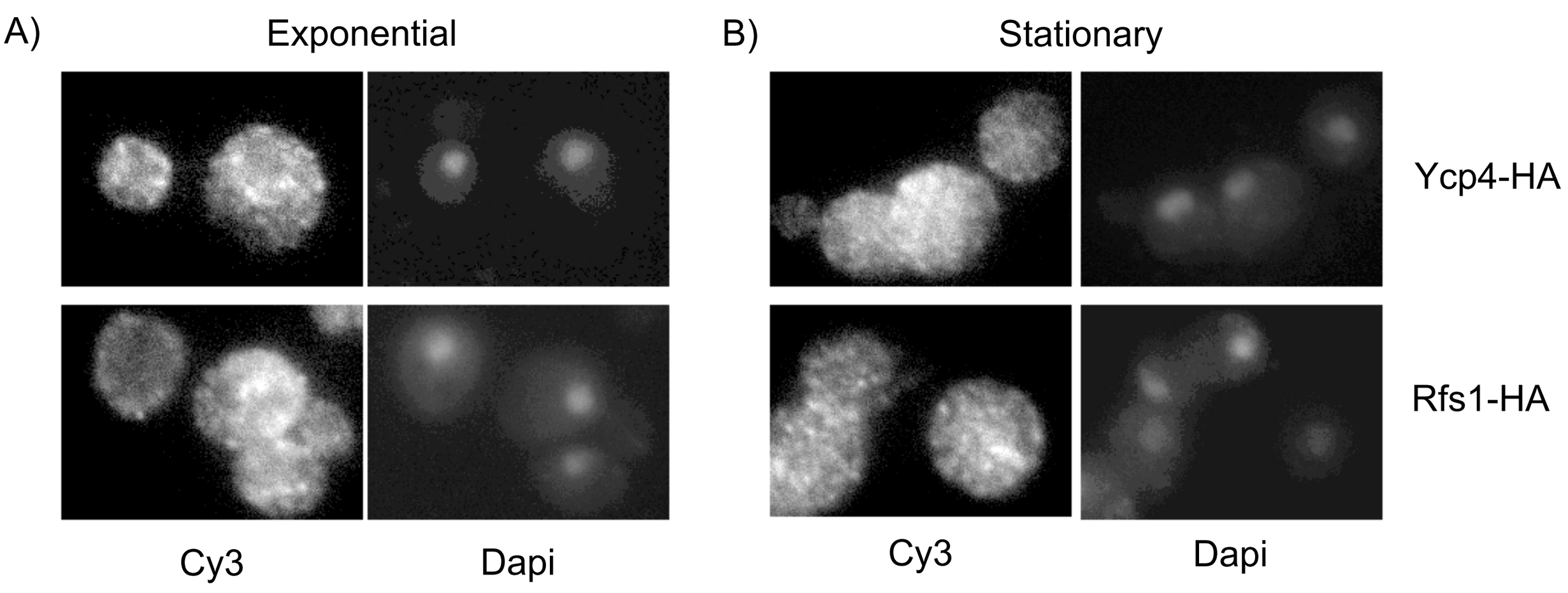
60
50
40
30
20
10
0
0
50
100
150
200
250
300
t (min)


A)
Exponential
B)
Stationary
Ycp4-HA
Rfs1-HA
Cy3
Dapi
Cy3
Dapi